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Enterotoxigenicity of Multidrug Resistant *Staphylococcus aureus* Isolated from Dry Catfish Sold in Some Open Markets in Zaria - Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Authors GOA and JOO designed the study and wrote the protocol. Author JOO managed the experiments, performed the statistical analysis, managed the literature searches and wrote the first draft of the manuscript. Authors GOA and JAO managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to determine the enterotoxigenic potential of MDR *S. aureus* isolated from dry catfish sold in some open markets in Zaria, Nigeria.
Place and Duration of Study: Department of Pharmaceutical Microbiology, Ahmadu Bello University, Zaria and Central Laboratory, City Campus of the Usman Danfodio University, Sokoto, Nigeria, from December 2017 to March 2019.
Methodology: We collected five, each of multidrug resistant and susceptible *S. aureus* isolates from the Pharmaceutical Microbiology Laboratory of the Ahmadu Bello University, Zaria and confirmed them as *S. aureus.* Cell concentrates were harvested from 24 hours old Luria and Bertani (LB) pure broth culture *of S. aureus* isolates by centrifugation at 4°C, 8000 rpm (6800 × g)

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in a micro-centrifuge for two minutes at room temperature. We used ZymoBIOMICS™ DNA Miniprep Kits to extract the genomic DNA from the harvested cells. We used PCR to amplify the 16S RNA, *mecA* and the enterotoxin genes after an external optimization of the reaction to ensure a better amplification. Finally, we used exactly two percent agarose gel to resolve the PCR genomic DNA fragments with their primers.

Results: The results of the PCR and gel electrophoresis indicated that all the ten (10) strains of *S. aureus* tested for 16S rDNA gene where positive for the gene whereas only two strains (20%) out of the ten (10) were positive for *mecA* gene. Among the ten (10) strains of *S. aureus*, tested, four (40%) strains were positive for two or more staphylococcal enterotoxin genes. All but one phenotypic multidrug resistant isolates carried genes for enterotoxins whereas, phenotypically drug susceptible isolates showed no band for enterotoxins.

Conclusion: This study showed that enterotoxin genes were common amongst the antibiotics resistant strains, which indicated a possibility of a relationship between antibiotics resistance and enterotoxogenicity.

Keywords: Enterotoxins; catfish; S. aureus; MDR, superantigens.

1. INTRODUCTION

Several virulence factors implicated in the pathogenesis of *S. aureus* strains include thermonuclease, hyaluronidase, lipases, and hemolysisn [1], which are involved in tissue invasion of the host. Perhaps the most notable virulence factors associated with this microorganism are the heat-stable enterotoxins that cause the sporadic food-poisoning syndrome or foodborne outbreaks [2].

Staphylococcal enterotoxin (SE) proteins have remarkable ability to resist heat and acid. They are resistant denaturation by mild cooking of contaminated foods. They are pyrogenic and have ability to induce emesis and gastroenteritis as well superantigenicity. They are resistant to inactivation by gastrointestinal proteases. SEs belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins (PT), sharing common phylogenetic relationships, structure, function, and sequence homology.

SE cause toxic shock-like syndromes, food poisoning and several allergic and autoimmune diseases. Included within this group are the staphylococcal enterotoxins, two forms of toxic shock syndrome toxin (TSST), and a group of streptococci pyrogenic exotoxins [3]. Staphylococcal enterotoxins function, not only as potent gastrointestinal toxins but also as superantigens that stimulate non-specific T-cell proliferation. Although these are two separate functions localized on separate domains of the proteins, there is a high correlation between these activities and in most cases a loss of superantigen activity (because of a genetic mutation) results in loss of enterotoxic activity as well [4].

S. aureus is notorious for its ability to resist the activity of a number of antibiotics. Strains of methicillin-resistant *Staphylococcus aureus* (MRSA), which had been largely confined to hospitals and long-term care facilities are emerging in the community [5]. The development of resistance both in human and animal bacterial pathogens has been associated with the extensive therapeutic use of antimicrobials or with their administration as growth promoters in food animal production [6]. MRSA cause most of the nosocomial *S. aureus* infections [7] which have become a widely recognized cause of morbidity and mortality throughout the world.

The paucity of literature in this area of knowledge as well as the love for dry fish consumption in the study area provided the bases for this study to determine the enterotoxigenic potential of MDR *S. aureus* isolated from dry catfish sold in some open markets in Zaria, Nigeria.

2. MATERIALS AND METHODS

2.1 Collection and Confirmation of *S. aureus* Isolates

We collected five, each of multidrug resistant and susceptible *S. aureus* isolates from the Pharmaceutical Microbiology Laboratory of the Ahmadu Bello University, Zaria and subjected to Gram reaction, catalase production, and agglutination reaction. We employed the detection of 16S rRNA in addition to Microgen Staph ID kits to confirm the isolates as *S. aureus.* No reference isolate of *S. aureus* was used in this study however, molecular grade water was used as negative control. Molecular analysis was carried out to detect the presence of *mecA* and four (4) enterotoxin genes.

2.2 Bacterial Cell Preparation

Pure colonies of the isolates were inoculated in 5 mL of Luria and Bertani (LB) broth and incubated overnight at 37°C for 24 h. Bacterial cells were harvested by centrifugation at 4°C, 8000 rpm (6800 × g) in a micro-centrifuge for two minutes at room temperature in an Eppendorff's tube, the supernatant was discarded, and cells harvested. We repeated the step above for higher yield of the cells [8].

2.3 Genomic DNA Extraction

We used the method described by Zymo Research Protocol to extract the genomic DNA [8]. The harvested cell pellet was dislodged and 200 µL of deionized water was added and mixed thoroughly by vortexing. Exactly 400 µL of the lysis solution was added to the mixture and mixed. The mixture was further incubated at 70°C for 15 minutes until the cells were completely lysed and appearing viscous to prevent clogging of the zymo-spin column. Exactly 400 µL supernatant was transferred to a Zymo-spin™ IV spin filter in collection tube and centrifuged at 7000 rpm for one minute. About 1,200 µL of DNA binding buffer was added to the filtrate in the collection tube from the preceding step. Exactly 800 µL of the mixture from the step above was transferred to a zymo spin IIC Column in a new collection tube and centrifuged at 10,000 × g for one minute. The flow-through in the step above in the collection tube was discarded and the step above repeated. A measure of 200 µL DNA prewashed buffer was added to zymo spin column in a new collection tube and centrifuged at 10,000 × g for one minute. About 500 µL of DNA wash buffer was added to zymo spin column and centrifuged at 10,000 × g for one minute. The zymo spin was transferred to a clean 1.5 mL micro-centrifuge tube and 100 µL DNA elution buffer was added directly to the column matrix and centrifuged at 10,000 × g for one minute to elude the DNA [8].

2.4 Polymerase Chain Reaction (PCR) of Genomic DNA

We carried out the PCR of the extracted genomic DNA after an external optimization of the reaction to ensure a better amplification. The following procedure was followed: The thin walled PCR tubes were marked, and the following components added for each isolate for single reaction of 50 µL viz: 25 µL of Dream Taq™ PCR master mix was added in the PCR tube, 1.0 µL of forward primer, 1.0 µL of reverse primer, 7.0 µL of genomic DNA. Nuclease-free water (16 µL) was added in the PCR tube to make up a total volume of 50 µL. The samples were spun down as PCR is performed using the thermal cycling conditions as stated by Zymo Research, UK [8].

2.5 Agarose Gel Electrophoresis of PCR Products

We used exactly two percent agarose gel to resolve the PCR genomic DNA fragments with their primers. We obtained the primers used in this study from Zymo Research Corporation, United Kingdom as represented on Table 1.

Table 1. Nucleotide sequences, gene locations, and sizes of PCR products for the *S. aureus* gene-specific oligonucleotide primers used in this study

Gene	Primer	Oligonucleotide sequence (5′-3′)	Location within gene	Size of amplified product (bp)	References
Sea	GSEAR-1	GGTTATCAATGTGCGGGTGG	349–368	102	[9]
	GSEAR-2	CGGCACTTTTTTCTCTTCGG	431–450		
Seb	GSEBR-1	GTATGGTGGTGTAACTGAGC	666–685	164	
	GSEBR-2	CCAAATAGTGACGAGTTAGG	810–829		
Sec	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	432–455	451	
	GSECR-2	CACACTTTTAGAATCAACCG	863–882		
Sed	GSEDR-1	CCAATAATAGGAGAAAATAAAAG	492–514	278	
	GSEDR-2	ATTGGTATTTTTTTTCGTTC	750–769		
See	GSEER-1	AGGTTTTTTCACAGGTCATCC	237–257	209	
	GSEER-2	CTTTTTTTTCTTCGGTCAATC	425–445		
mecA	GMECAR-1	ACTGCTATCCACCCTCAAAC	1182–1201	163	
	GMECAR-2	CTGGTGAAGTTGTAATCTGG	1325–1344		
16S rDNA		CAG CTC GTG TCG TGA GAT GT AAT CAT TTG TCC CAC CTT CG		420	

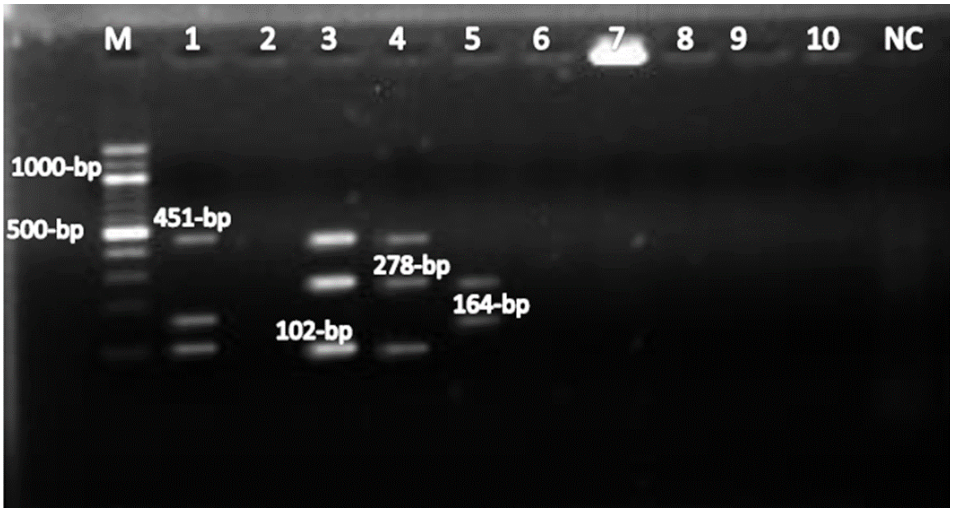

Fig. 1. Agarose gel electrophoresis patterns showing multiplex PCR amplification products for selected *S. aureus* enterotoxin genes. Lane M, DNA molecular size marker (100-bp ladder); Lanes 1 – 10, *Staphylococcus aureus* strains investigated; Lane NC, molecular grade water used as negative control

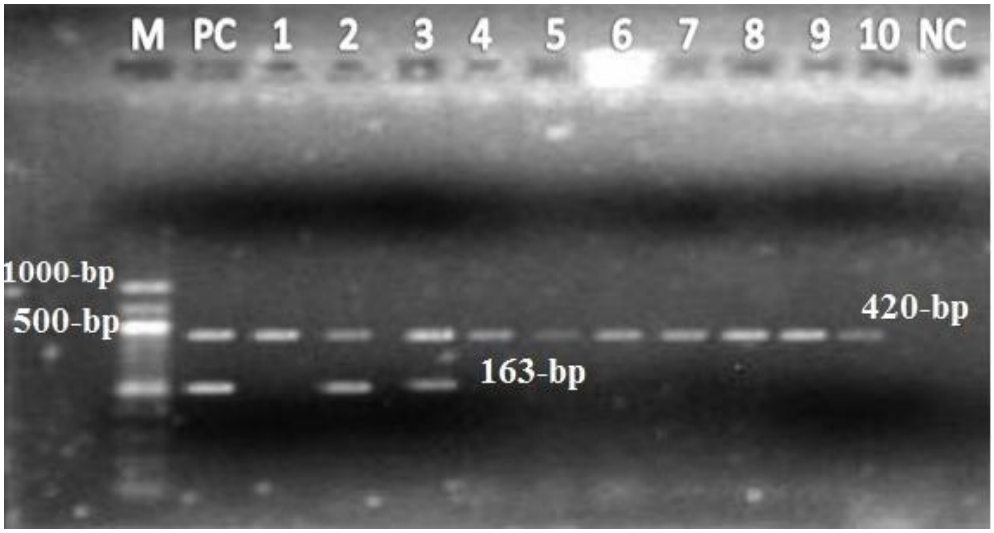

Fig. 2. Agarose gel electrophoresis patterns showing PCR amplification products for *mecA* and 16S rDNA genes. Lane M, DNA molecular size marker (100-bp ladder); Lane PC, Positive control strain for *mecA* and 16S rDNA; Lanes 1 – 10, *Staphylococcus aureus* strains investigated; Lane NC, molecular grade water used as negative control

3. RESULTS

3.1 Multiplex PCR Detection of Selected Enterotoxin Genes

Among the ten (10) strains of *S. aureus*, tested, four (40%) strains were positive for two or more staphylococcal enterotoxin genes. All but one strain that showed phenotypic resistance to multi antibiotics carry the genes for enterotoxins whereas, those that were phenotypically susceptible to antibiotics tested did not show any band for enterotoxins (Fig. 1). Three strains each showed bands corresponding to staphylococcal enterotoxin (se) a, c and d genes while two strains showed bands corresponding to staphylococcal enterotoxin b (*seb*) gene. We did not detect staphylococcal enterotoxin e (*see*) from any of the *S. aureus* in this study (Fig. 1).

3.2 Multiplex PCR Detection of mecA and 16S rDNA Genes

All the 10 strains of *Staphylococcus aureus* tested in this study harbour 16S rDNA gene whereas only two strains (20%) were positive for *mecA* gene (Fig. 2).

4. DISCUSSION

In this study, 40% of strains studied were positive for two or more staphylococcal enterotoxin genes. All but one strain that showed phenotypic resistance to multi antibiotics carried genes for enterotoxins whereas, those that were phenotypically susceptible to antibiotics tested did not show any band for enterotoxin genes. This result is in agreement with the study of Varshney et al. [10] the carriage of more than one enterotoxin gene in the clinical isolates of *S. aureus*. In fact, in their study, the median number of enterotoxin genes was five and some of the isolates contained up to 12 genes coding for different enteroxins as reported. Enterotoxin genes were not detected in the antibiotic susceptible *S.* aureus in this study. This may be because most genes coding for staphylococcal enterotoxins are located on mobile elements such as plasmids, bacteriophages or pathogenicity islands, which may also code for other factors such as resistance. In this study, the most common staphylococcal enterotoxin genes encountered were *sea, sec* and *sed* with percentage prevalence of 30% each. This report is similar to that of Pichuk et al. [11] who reported *sea* and *seb* as the commonest encountered enteroxins, however the report of this present study contradicted Pinchuk et al. [11] since *seb* was the least encountered.

It is important to note that all the staphylococcal enterotoxin genes detected in this study if expressed have significant roles in food poisoning and as biological warfare especially seb which is more virulent in comparison with others [11,12]. SED is the second most common staphylococcal toxin associated with food poisoning worldwide, and that only very small amounts of this toxin were needed to induce food poisoning [13]. SEE causes food poisoning in some cases [11].

We detected methicillin resistance gene (*mecA*) in two out the five MDR isolates tested by PCR. According to Prabhu et al. [14], inhibition of ≤ 19 around cefoxitin disc indicates MRSA. However, in this study, phenotypically, there was no MRSA encountered but with PCR analysis two strains of *S. aureus* studied showed the carriage of *mecA* gene responsible for methicillin resistance. This result is in line with the observation of Kalhor et al. [15] who suggested that heterogeneous resistance among MRSA strains may contributes to the failure of phenotypic methods to detect *mecA* positive strains, which are mixed with the more frequent negative ones. The low distribution of *mecA* gene in this study suggests that β-lactam antibiotics may still be efficient in the control of isolates from this source if implicated in infections and diseases.

All the isolates tested were positive for 16S rDNA, confirming that they belong to the same genus of Staphylococcus. Unlike phenotypic identification, 16S rDNA sequencing provides accurate identification of isolates with a typical phenotypic characteristic [16].

5. CONCLUSION

Out of the multidrug resistant isolates tested for the carriage of enterotoxin genes, only one was devoid of any enterotoxin genes. These results are serious causes for concern for public health and the safety of our ready-to-eat food items sold in the area and required urgent attention.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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